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(54) Title: A PROCESS FOR THE GENE MANIPULATION OF PLANT CELLS, RECOMBINANT PLASMIDS, RECOMBINANT BACTERIA, PLANTS

(57) Abstract

A process for the gene manipulation of cells of plants which, in essence, cannot be transformed by *Agrobacterium tumefaciens* with integration by T-DNA, which comprises introducing foreign DNA into the plant cells by infecting the plant cells with one or more recombinant *Agrobacterium tumefaciens* strains which contain foreign DNA to be transferred to the plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and are capable of transferring this foreign DNA to the plant cells, the foreign DNA located between the left- and right-hand ends of T-DNA comprising: (A) a transposase gene located in an expression cassette active in the plant cells but not beteween the left- and right- hand transposon ends necessary for integration in the DNA of the plant cells, and (B) a recombinant transposon comprising the left- and right- hand transposon ends necessary for integration in the DNA of the plant cells with an intermediate DNA fragment to be integrated in the DNA of the plant cells, but containing no DNA fragment which can lead in the plant cells to expression of an active transposase.

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Title: A process for the gene manipulation of plant cells, recombinant plasmids, recombinant bacteria, plants

This invention relates to a process for the gene manipulation of plant cells, which comprises introducing foreign DNA into plant cells by infecting the plant cells with one or more recombinant Agrobacterium tumefaciens strains, which contain foreign DNA to be transferred to the plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and are capable of transferring this foreign DNA to the plant cells.

The invention further relates to a recombinant plasmid consisting of a bacterial vector plasmid and at least one DNA insert, and to a recombinant Agrobacterium tumefaciens strain, which contains foreign DNA to be transferred to plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and is capable of transferring this foreign DNA to the plant cells.

Finally, the invention also relates to the plants deriving from the transformed cells and to the parts consisting of one or more cells or products thereof.

The Agrobacterium tumefaciens technology for the gene manipulation of plants, which technology has strongly developed and has been applied on a large scale since its first discovery, is in principle still limited, despite all progress made through the years, to the application in specific, substantially dicotyl plant species, such as tobacco, tomato, colza etc. Up to the present time a successful transformation of monocotyl plant species has essentially only been described for some Liliaceae, namely Asparagus (Bytebier et al, Proc. Natl. Acad. Sci. USA 84, 5345-5349, 1987) and Narcissus (Hooykaas et al, Nature 311, 763-764, 1984), some Iridaceae (Graves et al, J. Bacteriol. 169, 1745-1746, 1987), some Dioscoreaceae (Schafer et al, Nature 327, 529-532, 1987), and some Gramineae, namely Zea mays (Graves and Goldman, Plant Mol. Biol. 7, 43-50, 1986) and Oryza sativa (Raineri et al, Biotechnology 8, 33-38, 1990).

Apart from these exceptions, one has not yet succeeded in realizing by means of the Agrobacterium tumefaciens technology an efficient transformation of monocotyl plant species, including the important cereals such as maize, wheat, barley, rye, oat, rice etc. and in producing stably transformed monocotyl plants. In a broader sense this applies to many species of plants, not only to the cereals belonging to the group of monocotyl plants but also to grasses such as ryegrass and fescue, leguminous plants such as leek, onion and garlic, ornamental plants such as tulipe, hyacinth, bromeliad, iris, orchid etc., and other plant species such as conifer etc.

There seem to be indications that the problem is not due to the transfer of the DNA. Thus it has been found possible to transmit viral genomes encoded in the form of DNA by means of Agrobacterium tumefaciens to maize (Grimsley et al, Biotechnology 6, 185-189, 1988) and wheat (Dale et al, Seventh International Wheat Genetics Symposium 1, 719-722, 1988). Perhaps the problem is therefore due to the integration of the DNA in the genome: the integration system which Agrobacterium tumefaciens bacteria successfully use in dicotyl plants might not or not efficiently work in monocotyl plants. Generally, however, it is assumed that a poor transfer of the T-DNA to the plant is responsible for the failure of the technology in monocotyl plants.

In order yet to obtain the desired transformation in monocotyl plants, different alternative transformation techniques have been conceived and developed in which no use is made of Agrobacterium tumefaciens bacteria for transmitting DNA to the plant cell. Examples thereof are microinjection with DNA, bombardment with DNA-carrying particles, electroporation of protoplasts with DNA etc.

These techniques have been successfully employed for the introduction of DNA into plant cells, but generally only a passing expression of the genes introduced could be observed.

As regards the use of integration systems, the selection made so far concerns either homologous genetic recombination,

which is a hardly efficient system and remains limited in practice to cells in meiosis, or transposition, which is an efficient system and is also active during the normal cell cycle but has the drawback that the genes introduced are not stable in the first generation and that it is necessary to wait one or more generations before the transposon and the genes introduced via transposition are separated by mendelizing out.

Transposons are, in fact, genetic elements which can move within the genome of the host cells in which they are normally present. They have virtual ends, repeated border sequences which are in general invertedly repeated and may be rather different in length. At the location of their insertion into the genome a piece of host sequence is found back directly repeated to the left and the right of the transposon sequence. There is generally no specific selection of the location of insertion. In case of excision either the original sequence is restored or a deletion occurs or part of the sequence remains at the relevant location (insertion). In its simplest form a transposon consists of a transposase gene located between terminal sequences which contain transposase binding sites and optional binding sites for other host enzymes and terminate with the repeated border sequence. These terminal sequences, together with the directly repeated sequences of the target DNA to the left- and right-hand of the border sequence, will also be referred to hereinbelow as left- and right-hand transposon ends.

The present invention provides a process which removes the main objections of the known methods and gives a very efficient and stable transformation of cells of monocotyl plants, which has not been found possible so far. For this purpose use is made according to the invention of the efficient transfer system of Agrobacterium tumefaciens bacteria in combination with the efficient integration system of transposons.

The invention relates in a first aspect to a process for the gene manipulation of cells of plants which, in essence, cannot be transformed by Agrobacterium tumefaciens with integration by T-DNA, with foreign DNA being introduced into the plant cells by infecting the plant cells with one or more recombinant Agrobacterium tumefaciens strains which contain foreign DNA to be transferred to the plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and are capable of transferring this foreign DNA to the plant cells, the foreign DNA located between the left- and right-hand ends of T-DNA comprising:

(A) a transposase gene located in an expression cassette active in the plant cells but not between the left- and right-hand transposon ends necessary for integration in the DNA of the plant cells, and

(B) a recombinant transposon comprising the left- and right-hand transposon ends necessary for integration in the DNA of the plant cells with an intermediate DNA fragment to be integrated in the DNA of the plant cells but containing no DNA fragment which can lead in the plant cells to expression of an active transposase.

By the words "plants that, in essence, cannot be transformed by Agrobacterium tumefaciens with integration by T-DNA" are meant all those plant species in which Agrobacterium tumefaciens is not capable of effecting an efficient integration of the T-DNA in the plant genome, which roughly means almost all the non-dicotyl plants. The invention is therefore eminently suited for the gene manipulatin of monocotyl plants and other non-dicotyl plants, preference being given according to the invention to gene manipulation of plant cells of cereals such as maize, wheat, barley, rye, oat, rice etc., grasses such as ryegrass and fescue, leguminous plants such as leek, onion and garlic, ornamental plants such as tulipe, hyacinth etc. and other plant species such as conifer etc.

According to the invention there is used a transposase gene which is not located between the left- and right-hand transposon ends necessary for integration in the DNA of the plant cells. According to the invention there is further used a recombinant transposon containing no DNA fragment which can lead in the plant cells to expression of an active transposase. This means that the recombinant transposon is an inactive transposon. Consequently, a desirable high stability of the transformed plants can be obtained. The drawback of instability normally connected with the use of transposons is, in fact, the result of the presence of a complete (or active) transposon, i.e. a transposon with a transposase gene located between the transposon ends. This gene is then co-incorporated with the transposon in the plant genome and may therefore give rise for several generations to the undesirable instability. According to the present invention there is used an active transposase gene, namely in order to ensure efficient integration of the foreign DNA located in a transposon in the DNA of the plant cell (in the plant cell the transposase gene leads to the formation of transposase which, in cooperation with nuclear factors of the plant, is capable of effecting efficient integration of the transposon in the genome) but if this transposase gene is not within a transposon, in conformity with the preferred embodiment, it is not itself efficiently integrated in the genome in case of transformation of non-dicotylidons, such as in particular monocotylidons. This means that the desired stability is obtained already in the first generation of plants.

The transposase gene preferably consists of a genomic clone of the transposase gene, i.e. of genomic DNA which comprises not only the encoding exons but also the introns normally present. Although the use of a cDNA coding for the transposase is also possible, practice has shown that it gives a substantially less efficient integration of the recombinant transposon. The use of a genomic transposase gene does not mean that the natural promoter of the transposase gene should

be used too. Practice has shown that it is very advantageous to use, instead of the natural promoter, another promoter active in plants, in particular a promoter that can effect efficient expression in cells from which germinative cells develop. A promoter suitable for this purpose is the known CaMV 35S promoter. The use of the pTR1' or pTR2' promoter is also posible and favorable if, as is also preferably done according to the invention, transfer occurs by microinjection with the apical meristem being mechanically damaged. As a result, the wound-inducible regulators of gene expression for these promoters are present in the meristematic cells. The use of such a construct of a genomic transposase gene under control of another promoter such as the CaMV 35S, the pTR1' or the pTR2' promoter, therefore constitutes a highly preferred embodiment of the invention.

According to the invention it is particularly preferred that between the left- and right-hand transposon ends the recombinant transposon comprises at least one foreign gene located in an expression cassette active in the plant cells. By this is meant any gene that is to be incorporated in the genome of the plant for whatever reason. It may be a gene coding for a desirable property of the plant, such as resistance to herbicides, to pathogens, such as specific fungi and insects, or to environmental factors, or properties, such as (in ornamental plant cultivation) a specific flower color, flower scent, flower size etc., or properties such as (in case of food crop) better nutritional properties, a higher yield etc. It may also be a gene coding for a substance one wishes to produce on a large scale by cultivating a plant capable of forming the substance by means of gene manipulation. The gene to be introduced into the plant may therefore be a gene within the scope of plant breeding or a gene within the scope of biological production of specific products (proteins and enzymatic products, DNA, RNA etc.).

It is also possible that between the left- and right-hand transposon ends the recombinant transposon contains a

promoter active in plants, instead of a foreign gene in an expression cassette active in plants. Consequently, it becomes possible to alter the expression behavior of specific genes and thus the phenotype.

It is further preferred that also one or more selectable marker genes suitable for selection of transformed bacteria and located in an expression cassette active in the bacteria are located between the left- and right-hand ends of T-DNA. This enables selection of Agrobacterium tumefaciens bacteria with the desired DNA constructs. Marker genes suitable for this purpose are known to those skilled in the art. These are often genes the expression of which leads to a resistance to specific antibiotics, such as resistance to kanamycin, chloramphenicol, ampicillin, tetracycline, spectinomycin etc.

It is particularly preferred here that one or more selectable marker genes suitable for selection of transformed bacteria and located in an expression cassette active in bacteria form part of the recombinant transposon. This preferred embodiment has the advantage that the marker genes coupled to the foreign gene are co-incorporated in the genome of the plant so that in cases that as a result of the incorporation of the recombinant transposon in a gene of the host the transformed plant shows another phenotype (which is not due to the introduced genetic information as such) or that the foreign gene introduced proves to function differently in later generations than in the first generations (e.g. as a result of mutation) the recombinant transposon can be cloned for further examination from the plant genome into bacteria again by means of selection with the marker gene active in bacteria.

It is further preferred that also one or more selectable marker genes suitable for selection of transformed plant cells and located in an expression cassette active in the plant cells are located between the left- and right-hand ends of T-DNA. Also here it applies that many marker genes suitable for this purpose are known to those skilled in the art. Thus

the resistance to, e.g., antibiotics or herbicides can be mentioned as a property which, as is known to those skilled in the art, can be used for a selection of successfully transformed plants.

It is particularly preferred here that one or more selectable marker genes suitable for selection of transformed plant cells and located in an expression cassette active in the plant cells form part of the recombinant transposon. This preferred embodiment has the advantage that the marker genes are co-incorporated in the genome of the plant so that also later generations can be selected on the basis of the properties encoded by the marker genes.

A first variant of the process according to the invention is characterized in that the plant cells are infected with a mixture of two different recombinant A. tumefaciens strains, one of which comprises between the left- and right-hand ends of T-DNA the transposase gene located in an expression cassette active in the plant cells and the other the recombinant transposon between the left- and right-hand ends of T-DNA. This variant has the advantage of having a wide range of applications, i.e. there can always be used the same Agrobacterium strain supplying the transposase gene and only the second strain containing the recombinant transposon must be constructed for each new gene. In this second strain there is used a construct in which a multiple cloning site is located between the transposon ends so as to have a great freedom of choice with respect to the DNA fragments to be incorporated in the transposon.

In practice, in this first variant according to the invention both the first and the second A. tumefaciens strain will comprise between the left- and right-hand ends of T-DNA at least one marker gene for selection of transformed bacteria and at least one marker gene for selection of transformed plant cells. It is preferred here that at least the marker genes for selection of transformed plant cells which carry the two bacterial strains within the T-DNA ends are different from

each other so as to recognize plant cells successfully transformed by the two strains.

An alternative embodiment of the process according to the invention is characterized in that the plant cells are infected with one recombinant A. tumefaciens strain which comprises between the left- and right-hand ends of T-DNA both the transposase gene located in an expression cassette active in the plant cells and the recombinant transposon. This variant has the advantage of more chance of success of the transformation, i.e. the desired DNA transfer and integration will have taken place in a larger part of the treated plant cells.

In practice, in this second variant according to the invention the A. tumefaciens strain will comprise between the left- and right-hand ends of T-DNA at least one marker gene for selection of transformed bacteria and at least one marker gene for selection of transformed plant cells.

The process according to the invention is of special importance to the transformation of plant cells of a monocotyl plant species such as maize, wheat, barley, rye, oat, rice etc. An efficient transformation of such crops has not been found possible so far.

The invention can also be used, however, for the purpose of altering phenotypic characteristics of plants resulting from the presence of inactive transposons in one or more genes of the plant by removing such a transposon from the gene in which it is contained. For this purpose the invention provides a process for the gene manipulation of cells of plants which, in essence, cannot be transformed by Agrobacterium tumefaciens with integration by T-DNA, which comprises introducing foreign DNA into the plant cells by infecting the plant cells with a recombinant Agrobacterium tumefaciens strain which contains foreign DNA to be transferred to the plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and is capable of transferring this foreign DNA to the plant cells, which process is characterized in that plant

cells are infected which contain one or more inactive transposons but no active transposase gene and that the foreign DNA located between the left- and right-hand ends of T-DNA comprises a transposase gene located in an expression cassette active in the plant cells, which cassette is not located between the left- and right-hand transposon ends necessary for integration in the DNA of the plant cells.

The present invention is not subject to special limitations regarding the method of contacting the plant cells to be transformed with the Agrobacterium tumefaciens bacteria. In order to be able to obtain several generations of transformed plants, it is particularly preferred, however, that cells of the apical meristem in germinating seeds are infected with the bacteria.

In connection with practical advantages, in particular a high production per time unit and a high precision, it is preferred that the infection is carried out by means of needleless high-pressure injection. By this is meant a bombardment directed to cells of the apical meristem under high pressure of germinating seeds of the plants to be transformed, with a liquid containing the Agrobacterium tumefaciens bacteria. Instruments suitable for such a bombardment, such as a high-pressure "pistol", are known per se.

The invention, however, is not limited to such a method and comprises the possibility of carrying out the infection by means of microinjection. Moreover, also other meristematic or embryogenic tissues or cells can be used for introducing a foreign gene.

In another aspect the invention relates to a recombinant plasmid consisting of a bacterial vector plasmid and at least one DNA insert, which recombinant plasmid is characterized in that at least one DNA insert comprises a transposase gene located in an expression cassette active in plant cells, which expression cassette active in plant cells and filled with a transposase gene is not located between the left- and right-

hand transposon ends necessary for integration in the DNA of plant cells.

The invention further relates to a recombinant plasmid consisting of a bacterial vector plasmid and at least one DNA insert, which recombinant plasmid is characterized in that at least one DNA insert comprises a recombinant transposon which contains the left- and right-hand transposon ends necessary for integration in the DNA of plant cells but no DNA fragment which can lead in plant cells to expression of an active transposase.

A preferred embodiment of such a recombinant plasmid is characterized in that between the left- and right-hand transposon ends the recombinant transposon comprises one or more restriction endonuclease recognition sequences suitable as cloning site. Such a plasmid has a wide range of applications as a carrier for genes that are to be introduced into the genome of plants.

A special preferred embodiment of such plasmids is characterized in that the recombinant transposon comprises between the left- and right-hand transposon ends at least one foreign gene located in an expression cassette active in plant cells.

In such recombinant plasmids it is further preferred that the recombinant transposon comprises between the left- and right-hand transposon ends one or more selectable marker genes suitable for selection of transformed plant cells and located in an expression cassette active in plant cells, and that the recombinant transposon comprises between the left- and right-hand transposon ends one or more selectable marker genes suitable for selection of transformed bacteria and located in an expression cassette active in bacteria.

In yet another aspect the invention provides a recombinant Agrobacterium tumefaciens strain which contains foreign DNA to be transferred to plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and is capable of transferring this foreign DNA to the plant

cells, which strain is characterized in that a transposase gene located in an expression cassette active in plant cells is located between the left- and right-hand ends of T-DNA, which expression cassette active in plant cells and filled with a transposase gene is not located between the left- and right-hand transposon ends necessary for integration in the DNA of plant cells.

As regards this aspect, the invention also provides a recombinant Agrobacterium tumefaciens strain which contains foreign DNA to be transferred to plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and is capable of transferring this foreign DNA to the plant cells, which strain is characterized in that between the left- and right-hand ends of T-DNA a recombinant transposon is located, which comprises the left- and right-hand transposon ends necessary for integration in the DNA of plant cells but no DNA fragment which can lead in plant cells to expression of an active transposase.

For such a recombinant Agrobacterium tumefaciens strain it applies again that the recombinant transposon may comprise between the left- and right-hand transposon ends at least one foreign gene located in an expression cassette active in plant cells and/or one or more selectable marker genes suitable for selection of transformed plant cells and located in an expression cassette active in plant cells, and/or one or more selectable marker genes suitable for selection of transformed bacteria and located in an expression cassette active in bacteria.

A special embodiment of such a recombinant Agrobacterium tumefaciens strain is characterized in that also a transposase gene located in an expression cassette active in plant cells is located between the left- and right-hand ends of T-DNA, which expression cassette active in plant cells and filled with a transposase gene is not located between the left- and right-hand transposon ends necessary for integration in the DNA of plant cells.

The invention is finally also contained in plants and parts consisting of one or more cells or products thereof, which plants derive from plant cells transformed by the process according to the invention. By parts and products of plants is meant, e.g., the bulb, the tuber or the root, the flowers, the seed and the meal made therefrom etc.

The invention will be further explained with reference to the accompanying drawings, which diagrammetically show in

Fig. 1 a T-DNA construct comprising both a foreign gene X included in an inactive transposon and a transposase gene not located in an active transposon,

Fig. 2a a T-DNA construct comprising a foreign gene X included in an inactive transposon,

Fig. 2b a T-DNA construct which can be used in combination with that of Fig. 2a and comprises a transposase gene not located in an active transposon,

Fig. 3a a T-DNA construct comprising a foreign gene X included in an inactive transposon which comprises a marker gene interrupted by a second inactive transposon for selecting transformed plant cells,

Fig. 3b a T-DNA construct which can be used in combination with that of Fig. 3a and comprises a transposase gene not located in an active transposon,

Fig. 4a a T-DNA construct comprising a foreign gene X included in an inactive transposon, which foreign gene X is interrupted by a second inactive transposon,

Fig. 4b a T-DNA construct which can be used in combination with that of Fig. 4a and comprises a transposase gene not located in an active transposon,

Fig. 5a the structure of the recombinant plasmid pMH10, pMH10-LTS and pMH10-DsB, respectively a plasmid having a complete transposon, a transposase gene with only the left transposon end and a transposon without transposase gene,

Fig. 5b an example of a T-DNA construct with plasmid pMH10-LTS according to the invention,

Fig. 6a the structure of the recombinant plasmid pMH10Ds15GUS,

Fig. 6b an example of a T-DNA construct with this plasmid according to the invention,

Fig. 7 the structure of the recombinant plasmid pMH10Ds34CAT,

Fig. 8 the structure of the recombinant plasmid pMH10Ds12IAA,

Fig. 9 the structure of the recombinant plasmid pMH11Ds4IPTkan,

Fig. 10 the structure of the recombinant plasmid pUCSSAc9Ds19, suitable for cloning foreign genes between the transposon ends of Ac9,

Fig. 11a the structure of the recombinant plasmid pUCSS19tpnAc9P35S,

Fig. 11b the structure of the recombinant plasmid pUCSS19tpnAc9PTR1,

Fig. 11c a T-DNA construct with the recombinant plasmid pUCSS19tpnAc9P35S according to the invention.

Fig. 12a the structure of the recombinant plasmid pUCSSDs19BTKA,

Fig. 12b a T-DNA construct with this plasmid according to the invention,

Fig. 13a a cointegrate plasmid with the constructs pMH11Ds4IPTkan and pUCtpnAc9P35S, and

Fig. 13b an example of a T-DNA construct with the cointegrate plasmid pMH11Ds4IPTkan/pUCtpnAc9P35S.

In the drawings the symbols have the following meanings:

X represents a foreign gene located in an expression cassette, not separately shown, active in plant cells; if the gene is interrupted by an insert, the component parts of the gene are indicated by X' and X", respectively;

S1 represents a marker gene which can be used for the selection of transformed plant cells; here, too, the component parts are indicated by S1' and S1", respectively, when the gene is interrupted by an insert;

S2 and S3 also represent marker genes suitable for the selection of successfully transformed plant cells, further referred to as plant selection genes;

s1 and s2 represent marker genes suitable for the selection of transformed bacteria, further referred to as bacteria selection genes;

tpn represents a genomic clone of a transposase gene located in an expression cassette, not separately shown, which can function in plant cells;

LTA and RTA represent respectively the left end of T-DNA and the right end of T-DNA, which ends are necessary for transferring the T-DNA by the Agrobacterium tumefaciens bacteria to the plant cell;

LTS and RTS represent respectively the left end of a transposon and the right end of an active or inactive transposon,;

Wx represents the sequence of the waxy gene of maize from which Ac9 was cloned;

Fig. 1 shows a T-DNA construct according to the invention, one plasmid containing between the T-DNA ends LTA and RTA both an inactive transposon having a foreign gene X and a plant selection gene S1 contained therein and a transposase gene tpn not located on an active transposon. The selection genes shown in Fig. 1 are facultative, for that matter, in particular one of the bacteria selection genes and one of the plant selection genes being dispensable, if required. As regards the transposase gene, it is important that it is not located within the transposon ends LTS and RTS. This does not mean that the transposon ends must be completely absent: often removal of (a significant portion of) one of the transposon ends will be sufficient to prevent efficient incorporation of the transposase gene in the genome of the transformed plant cells. Thus, for instance, one transposon end could be retained in Fig. 1 beside the transposase gene (see Fig. 5a).

Figs. 2a and 2b show a preferred embodiment consisting of an analogous construct, the elements being distributed over two different plasmids. The inactive transposon having a foreign gene X and a plant selection gene S1 contained therein is located on a first plasmid the T-DNA of which is shown in Fig. 2a. Fig. 2b shows the T-DNA of a second plasmid which contains a transposase gene and a plant selection gene S2. As shown in Figs. 2a and 2b, each of the two T-DNA constructs also contains a bacteria selection gene (s1 and s2, respectively).

For clarity's sake, reference is made to the fact that the present invention is in no way limited to the constructs concretely shown. Thus only one bacteria selection gene will easily do in the construct of Fig. 1. Also the location of the different elements can be varied, e.g. in Fig. 1 a bacteria selection gene can be placed in the transposon or the location of the plant selection genes can be altered with respect to the other elements. Also T-DNA constructs according to the invention may contain additional or other elements than are shown in the drawings, e.g. one or more additional foreign genes, one or more additional bacteria or plant selection genes etc.

Figs. 3a and 3b show a preferred embodiment resembling those of Figs. 2a and 2b. An inactive transposon having a foreign gene X and a plant selection gene S1 contained therein is located on a first plasmid, the T-DNA of which is shown in Fig. 3a. In this case, however, the plant selection gene S1 is interrupted by a second inactive transposon which, if required, contains its own plant selection gene S3 as shown in Fig. 3a. Fig. 3b shows the T-DNA of a second plasmid containing a transposase gene and a plant selection gene S2. This plasmid is identical with the plasmid shown in Fig. 2b.

Figs. 4a and 4b show a preferred embodiment corresponding to those of Figs. 3a and 3b, with the understanding that not plant selection gene S1 but foreign gene X is interrupted by the second inactive transposon.

Figs. 5 through 13 show DNA constructs which will be further illustrated by the Examples.

EXAMPLES

Example 1
Elimination of insertion mutations of sequences (Ds) derived from transposon Ac (Activator) in maize.

By introducing an active transposon Ac into the genome of maize by means of crossbreeding, the activity of this transposon can be observed in the next generations because of the development of mutations (insertion into a gene) or through the elimination of a mutation owing to Ac insertion (excision from a gene). In the example described here the Ac transposase gene is used for elimination of insertion mutations (in this case Ds) from genes of maize. In conformity with the above described technology Agrobacterium is used herein to introduce the transposase gene into cells of the apical meristem of maize.

The plasmid pMH10 (Fig. 5a) contains the PstI clone of transposon Ac9 in the waxy gene of maize (Pohlman et al, 1984, Müller-Neumann et al, 1984), a PstI-BamHI fragment of plasmid pBR322 (Bolivar et al, 1977) with a functional ori sequence for replication in gram-negative bacteria, but a non-functional amp and tet resistance gene and moreover a BamHI-PstI fragment of plasmid pR702 (Hedges and Jacobs, 1974, and Leemans et al, 1981) containing the sequence of an str/spc resistance gene. The plasmid was obtained from Laboratorium voor Genetika RUG, Ledeganckstraat 35, B9000 Ghent, Belgium.

In this plasmid the BamHI fragment containing the pBR32 sequence of the right Ac9(wx) transposon end was replaced by a BamHI fragment containing the complete pUC8 plasmid (Vieira and Messing, 1982). The resulting plasmid is called pMH101LTS (Fig. 5a). The pMH101LTS plasmid is transmitted by conjugation to a rif resistance Agrobacterium tumefaciens (A. tum.) C58

strain containing the Ti plasmid pGV3850 (Zambryski et al, 1983), in which pBR322 is cloned as a HindIII fragment into the T-DNA of pTi C58 from which, furthermore, all HindIII fragments were cut, except the Left Border (LB) fragment (HindIII-10) and the Right Border (RB) fragment (HindIII-23) containing the nopaline synthase gene (nos). The plasmid pGV3850 was obtained from Laboratorium voor Genetika RUG, Ledeganckstraat 35, B9000 Ghent, Belgium.

The method of conjugation was described by Van Haute et al (1983). By selection of transconjugants for rif, str/spc and amp resistance a cointegrate was selected, pMH101LTS being integrated between the borders of the T-DNA.

Maize lines obtained by inbreeding for at least ten years were made available, in the form of seed, by the firm of Clovis Matton, Avelgem-Kerkhove, Belgium. These lines contain no active transposon Ac, which, inter alia, could be proved by breaking down the DNA of young leaves with PvuII and hybridizing it with the internal HindIII fragment of Ac (Ac9(wx): bp: 1270-2877) : no hybridizing band was observed corresponding to the 2626 bp internal PvuII fragment of Ac (Ac9(wx): bp: 719-3345); different bands were found indeed but the smallest band exceeded 4 kb. Crossings with test lines for Ac activity have never shown transposition phenomena in the lines used. The lines are used for the production of commercial hybrids.

Seeds of ten maize lines were disinfected with NaClO 2% and then laid on sterile wet sand to germinate on sterile sand at 25°C in the dark. Three days after germination 50 nl induced A. tum. culture were integrated with the construction pMH10 LTS by genetic recombinantion between the borders of the T-DNA (Fig. 5b), injected into the apical meristem by microinjection.

The induction medium has been described by Vernade et al (1988). After one week at 20°C the young plants were cultured together with untreated plants in large pots in an air-conditioned greenhouse in a mixture of compost and sandy clay

in their normal growing season (May to October). Each plant was fertilized manually, both the plume and the ear being kept in small bags. Each plant was individually watered in the pot. The ears were harvested in September and October and after drying the seed was stored in paper bags during winter at 6°C. The next year the plants were sown on ear lines in the soil in the greenhouse and again fertilized manually and evaluated by corn growers for their characteristics both during growth and in surmaturity.

The following improvements over the original lines were observed in some ear lines:

- plume more branched
- 30 - 50 cm higher plants
- stronger stem
- significant increase in the number of grain rows per ear
- remains green for a longer time
- absence of fusariosis
- better anchoring by secondary roots

Seeds of plants having deviating features as well as of control plants were germinated in the laboratory under sterile conditions and DNA was prepared from the young leaves, after which the plants were planted in the greenhouse in large pots,

The DNA of each plant was then broken down such as for RFLP mapping with different restriction enzymes and hybridized with the internal HindIII fragment of Ac9. The control plants showed the same band pattern, as regards the prominent bands, as the original line. In plants with deviating features either disappearance of one or more fragments hybridizing with the DNA probe or the disappearance of a fragment and the appearance of a new fragment with a larger molecular weight was observed.

Example 2 (comparative example)

Insertion of a foreign gene into the genome of maize by using Agrobacterium tumefaciens as transfer system and transposon Ac as integration system.

For the purpose of comparison an active transposon Ac9(wx) is used here. By using the integration properties of the transposon Ac and the transfer properties of A. tum. a foreign gene located between the recognition sequences of the transposon can be incorporated in the genome of maize by means of transposase of the same transposon.

As described, two A. tumefaciens strains are used by way of example. The first contains the β-glucuronidase gene of Escherichia coli located between the left- and right-hand transposon ends of Ac9(wx), which, in turn, is located between the Left and Right Border (LB and RB) of the T-DNA on a non-oncogenic Ti plasmid. The second contains a complete transposon Ac9(wx) located between LB and RB of the T-DNA.

The A. tum. strain containing the transposon Ac9 in the T-DNA is shown in Fig. 5a. The other A. tum. strain was obtained in the following manner:
Plasmid pBI 221 contains the β-glucuronidase gene of E. coli (uidA) under the control of the CaMV 35S promoter (Cauliflower Mosaic Virus 35S RNA promoter) and the mRNA distal end of the nopaline synthase gene of the Ti plasmid of A. tum. T37. This plasmid was available from Dr R. Jefferson, c/o AFRC Institute, Colney Lane, Norwich NR4 7UA, U.K.

The plasmid pMH10 shown in Fig. 5a was cut with EcoRI and partially with HindIII and the 10.1 kb fragment was ligated with the 3 kb EcoRI-HindIII fragment of pBI 221 containing the sequence of the chimeric uidA gene of this last plasmid. The resulting plasmid pMH10Ds15GUS contains the chimeric uidA gene in a transcription orientation contrary to that of the (partially) deleted transposase gene.

A. tum. bacteria with this plasmid, introduced in the manner described in Example 1, show β-glucuronidase activity. The construction is shown in Figs. 6a and 6b.

The same maize lines described in Example 1 were used for the transformation. In three-day-old germs the apical meristem was infected by microinjection with 50 nl of a

mixture of the two above-described A. tum. strains induced for transfer. In a first phase some hundreds of germs were tested after 1 or 2 weeks for β-glucuronidase activity by incubating a piece of tissue containing the apical meristem with X-gluc (5-bromo-4-chloro-3-indolyl-D-glucuronide). In about 7.5% of the tissues blue staining occurred. With a dark field microscope it was clearly visible that the cells in the tissue cuts and not the remaining bacteria were the origin of the purple color. In particular small cells around the vascular bundles were stained. With an electron microscope the precipitate of dimeric bromo-chloro indole was found in cells which were removed from the place of injection up to ten cell layers. Sporadically, cut bacteria were visible which were only present in the extracellular spaces but showed no precipitate.

Other treated germs were grown in the greenhouse in a normal growing season, manually fertilized and the seed was harvested as described in Example 1.

During the winter months these seeds were germinated in sterile conditions and the proteins were isolated from the young leaves and subjected to polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. Beta-glucuronidase activity was detected by covering the gel with a thin agarose gel containing the substrate methyl-umbelliferyl-D-glucuronide which fluoresces in UV light if the β-glucuronidase enzyme causes release of methyl umbelliferone.

A fluorescent band was found in about 1% of the tested plants. DNA was prepared from leaves and pieces of roots of these plants and, as in RFLP mapping, hybridized with a 2.9 kb HindIII-EcoRI fragment containing the chimeric uidA gene. In the lane of the agarose gel containing maize DNA broken down with HindIII and EcoRI hybridization with the probe was found on a level with the place where about 3 kb fragments migrate.

Of young F1 plants pieces of leaves and roots were incubated in X-gluc as described by Jefferson (GUS gene fusion system user's manual, 1987). Blue stains, zones and sectors

were found in the pieces of tissues of PAGE-β-glucuronidase positive plants: around the vascular bundles, around the stomata, V-shaped segments of the roots, etc.

Light microscopic and electron microscopic analyses confirmed the expression of the β-glucuronidase enzyme in the plant cells.

This example clearly shows that by using an active transposon in combination with a chimeric transposon containing a foreign gene but no transposase gene between the transposon ends expression is only obtained in specific cells still containing the chimeric transposon, while most of the plant cells show no expression. The same phenomenon can be observed after transformation of plants such as tobacco which are efficiently transformed by Agrobacterium but in this case instability occurs after transformation with a chimeric transposon, both in the presence of an active transposon and in the presence of a transposase gene. This shows the difference between plants in which Agrobacterium can efficiently integrate foreign DNA in the plant genome and plants in which Agrobacterium cannot.

Example 3

Generation of mutations in barley by using a transposase gene and a chimeric transposon.

In this example the mutagenic properties of a transposon are used by integrating a chimeric transposon containing a plant selection gene but no transposase gene in the genome of barley by means of a transposase gene that is not located between transposon ends.

Use is made of two Agrobacterium strains for the transfer: the first strain contains the chimeric transposon between the T-DNA ends and the second strain contains the transposase gene between the T-DNA ends. This example corresponds with Figs. 3a and 3b.

The employed plasmids are pMH10Ds15GUS and pMH10LTS. As described before, they are conjugated from Escherichia coli to Agrobacterium and integration in pGV3850 selected by also adding spectinomycin to the medium.

Seeds of two- and six-rowed winter barley ear lines (F7-F8) were supplied by the plant breeding station of the firm of Clovis Matton, Avelgem-Kerkhove, Belgium.

Seeds of 12 ear lines were disinfected with 6% NaClO and the seeds laid on moist sand to germinate at 4°C. Germinating seeds were further incubated at room temperature under sterile conditions.

In 3-5-day-old seedlings the apical meristem was infected by microinjection with Agrobacterium strains containing pMH10Ds15GUS or pMH10LTS. Transfer was induced as described by Vernade et al (1988). Infections were conducted both with the strain containing pMG15 (control) and with the strain containing pMH10LTS (control) or with both of them (experiment). About 300 seedlings were treated per ear line: a hundred per experiment. Further control experiments were conducted on tobacco, for rapid tests of expression.

After 1 week at 22°C the young plants were cultured further in a greenhouse and the seed was harvested after self-pollination.

In the next generation the plants were sown again in a greenhouse and selected by barley growers for deviating features.

The following variants were traced:

- difference in height of the plant during growth
- difference in time of growth
- mutations from erectant to nutant or conversely
- difference in number of grains per ear
- difference in flattening
- better or poorer phytosanitary condition.

Of 37 ear lines with clear morphological or phytosanitary differences and 110 other ear lines with less

clear deviations five ripe ears were further examined for expression of the uidA gene in the chimeric transposon.

Nearly all the 37 plants with clear differences over the original ear line express the uidA gene to a greater or lesser degree. No β-glucuronidase activity could be demonstrated in the controls, except in the case of bacterial infection. Only 9 plants with less clear deviations show uidA expression.

In tobacco β-glucuronidase activity was found when the gene was present in the infection, with or without additional infection with the transposase gene.

Example 4

Construction of recombinant plasmids with plant selection genes or genes with industrial applications between left- and right-hand transposon ends in the plasmid pMH10.

In this example the construction of some plant selection genes was shown by using unique restriction sites in the sequence of Ac9 in the plasmid pMH10.

The chloramphenicol acetyl transferase gene in an expression cassette consisting of the 35S promoter of CaMV and the nos-terminator of the T-DNA of Agrobacterium tumefaciens is available as plasmid pCAMVCN from Pharmacia-LKB, Bromma, Sweden. The complete chimeric gene was cut as Xba1 fragment. The plasmid pMH10 was partially cut with Bcl1 (position 1530 in Ac9Wx) and BamH1 (position 4498 in Ac9Wx) and the 8.1 kb fragment was ligated back. This plasmid, called pMH10DsB, contains unique restriction sites in the remaining Ac9 sequence: Nsi1 (five sites at position 571, 972, 1003, 1183, and 1251 of Ac9Wx), Xho1 (at position 1125 of Ac9Wx) and Xba1 (at position 1257 of Ac9Wx) (Fig. 5a). The cat expression cassette was cloned into the unique XbaI site of Ac9. The resulting plasmid was called pMH10Ds34CAT (Fig. 7), conjugated to Agrobacterium and integrated in the plasmid pGV3850. By transformation of tobacco the expression of the cat gene was proved.

The genes iaaM and iaaH from the T-DNA of Agrobacterium tumefaciens Ach5 (Gielen et al, 1984) were cloned from the plasmids pGV814 (EcoR1-Cla1 clone of iaaM) (Budar et al, 1986) and pGV824 (HindIII clone of iaaH) (Budar et al, 1986). The complete construct with the genes iaaM and iaaH was first cloned into pBR322 (Bolivar et al, 1977) by a threefold ligation of the 3.6 kb pBR322 PstI-ClaI fragment, the 3.7 kb EcoR1-ClaI fragment of pGV814 and the 2.7 kb partial PstI-EcoR1 fragment of pGV824. From this was cloned the 6.4 kb PstI fragment in the NsiI sites (position 571 and 2881 of Ac9Wx) of pMH10. The resulting plasmid pMH10Ds12IAA (Fig. 8) was conjugated to Agrobacterium and integrated in the plasmid pGV3850 by additional selection with spectinomycin. The usability of the construction was tested by transformation of tobacco and expression of the iaa genes.

The gene ipt from the T-DNA of Agrobacterium tumefaciens C58 together with the bacterial nptII gene of transposon Tn5 of plasmid pG4L (Inzé et al, 1984) was cloned as a BamHI fragment in pUC19 and isolated therefrom as a partial Sph1-EcoR1 fragment of 3.7 kb and cloned into pMH11 cut with EcoR1 and partially with Sph1. pMH11 is a derivative of pMH10 obtained by cutting in the vector plasmid of pMH10 the 0.8 kb Sph1 fragment located between the str/spc resistance gene and the (partially deleted) tet resistance gene, blunt ending the ends with S1 nuclease and ligating back the vector. The PstI clone of Ac9Wx was again incorporated in the resulting vector in the unique PstI site. The orientation, however, is different from that in pMH10.

The resulting plasmid pMH11Ds4IPTkan (Fig. 9) was conjugated to Agrobacterium and integrated in pGV3850 by additional selection with spectinomycin and then with kanamycin. The usability of the construction was proved by transformation of tobacco and expression of the gene ipt.

Example 5

Construction of a recombinant transposon with a multiple cloning site between the transposon ends.

The SphI-KpnI fragment from mini-Sa, a derivative of pR702 (Leemans et al, 1983) containing the sm-sp adenosyl transferase gene encoding resistance to streptomycin and spectinomycin was blunt ended with T4 DNA polymerase and cloned into the plasmid pUC19 (Yanisch-Perron et al, 1985), blunt end cut with SspI. This plasmid was 4330bp and will be referred to hereinbelow as pUCSS19.

Both orientations of the sm/sp resistance gene are suitable. However, the work was further done with the plasmid in which the amp resistance gene and the sm/sp resistance gene have the same transcription orientation. pUCSS19 was blunt end cut with PvuII and both the 4030bp vector fragment and the 300bp MCS fragment were separated and stored.

The PstI clone of Wx-Ac9 was cut from pMH10 (Fig. 5a) and blunt ended with T4 DNA polymerase. This fragment was cloned into the 4030bp PvuII fragment of pUSS19. This plasmid was 8840bp and will be referred to hereinbelow as pUCSSAc9.

pUCSSAc9 was cut with NsiI and MscI and the NsiI end was blunt ended with T4 DNA polymerase. This fragment was 4980bp and contained the ends of transposon Ac9Wx. Into this was cloned the 300bp PvuII fragment of pUC19. One of the orientations is shown in Fig. 10. The plasmid was 5280 bp and will be referred to as pUCSSAcDs19. It can be used for cloning numerous genes with their expression cassette. It can be conjugated to Agrobacterium with plasmid pGV3850 as described in the first example by additional selection with spectinomycin.

Example 6.

Construction of recombinant plasmids containing plant selection genes or genes with industrial applications and located between the transposon ends of pUCSSAcDs19.

The delta endotoxin gene of the 42 MDa plasmid of Bacillus thuringiensis var. Berliner 1715 (Klier et al, 1982), the sequence of which was published by Höfte et al, 1986, was obtained from S.A. Solvay, Brussels, Belgium, as plasmid pBT424. The strain is available from the Institut Pasteur in Paris, France.

From pBT424 the BamH1-BclI fragment containing the start codon of the delta endotoxin gene and the first 1939 bp, necessary for expression of the toxin, was cut and cloned downstream of the pTR1' promoter of plasmid pPCV520 (Koncz and Schell, 1986), cut with BamH1 and BglII. The correct orientation was verified with BamH1 and PstI (0.7 kb in the correct orientation). The complete double expression cassette pTR1'-BTtox terminator gene 4/pTR2'-nptII terminator ocs was cut again with KpnI and partially with PstI as a 5.7 kb fragment and cloned into the corresponding restriction sites of pUCSSAcDs19. The terminator of gene 4 contains different stop codons in the three reading frames upstream of the polyadenylation signal.

The resulting plasmid (pUCSSDs19BTKA) (Fig. 12a) was conjugated to Agrobacterium and integrated in pGV3850 by additional selection with spectinomycin as described before (Fig. 12b).

Resistance to kanamycin could be shown after transformation to tobacco.

The construct was used for making maize lines resistant to Pyralidae, in the manner as described in Comparative Example 2, with the difference that use was made of a second Agrobacterium strain containing a transposase gene without transposon ends (pMH10LTS).

Example 7.

Construction of a recombinant plasmid containing the transposase gene of Ac under control of the CaMV 35S promoter or of the pTR1' promoter of Agrobacterium Ach5 T-DNA.

The plasmid pMH10 (Fig. 5a) was cut with PstI and the 4810bp Ac9 wx fragment, cloned into pUC19, cut wit PstI. This plasmid was 7500bp and will be referred to hereinbelow as pUC19Ac9wx. The work was further done with the orientation 1, namely the one in which the BamHI sites are remotest from each other.

The Ac9 wx fragment was cut again, as BamHI fragment. This fragment was partially cut with Cfr10I and two fragments were stored: the 3.4 kb Cfr10I fragment and the 4.2 kb BamHI-Cfr10I fragment. The first contained a transposase gene without a promoter and the second a transposase gene with a promoter. The 4.2 kb fragment was cloned into pUCSS19, cut with XmaI and BamHI. This plasmid (8.5kb) contained the normal transposase gene of Ac but no transposon ends (pUCSS19tpnAc9PAc9) and could be transmitted by conjugation to Agrobacterium and integrated in the T-DNA of pGV3850 by additional selection of spectinomycin resistance. The 3.4 kb fragment was cloned into pUC19, cut with XmaI, and the orientation was determined using the XbaI site in the MCS and in the transposase gene (position 1260 of Ac9Wx). This plasmid (7.7 kb) is called pUCSS19tpnAc9cds) and may serve for cloning new promoters upstream of the transposase gene.

For further use pUCSS19tpnAc9cds was taken in orientation 1, the XbaI fragment from this plasmid being 2.5 kb. This plasmid was cut with PstI and BamHI and the CaMV 35S promoter fragment from pBI221, cut with PstI and BamHI, was cloned into it.

The resulting plasmid is called pUCSS19tpnAc9P35S (Fig. 11a). The pTR1'2' double promoter of the T-DNA of Agrobacterium Ach5 (Velten et al, 1984) was obtained as a BamH1-PstI fragment from the plasmid pPCV520 (Koncz and Schell, 1986) and was cloned into the BamH1 and PstI sites of pUCSS19tpnAc9cds. The resulting plasmid (pUCSS19tpnAc9PTR1') contains the transposase gene under control of the pTR1' promoter (Fig. 11b).

Both plasmids can be conjugated, as described before, to

Agrobacterium and integrated in pGV3850 by additional selection for spectinomycin (Fig. 11c). The use of the pTR1' promoter has the advantage that in case of microinjection or bombardment of Agrobacterium under high pressure this promoter strongly expresses in the wounded tissue.

Example 8.
Construction of recombinant plasmids containing a chimeric transposon and a transposase gene without transposon ends.

Recombinants between a pMH10 derivative with a chimeric transposon and pUC19 derivatives with a chimeric transposase gene were selected in vivo in MC1061 bacteria after transformation with both plasmids and selection for ampicillin and spectinomycin, followed by alternating growth cycles with selection for ampicillin and then spectinomycin. This results in cultures in which recombinant cointegrates of pMH10 and pUC derivatives occur in 50% of the bacteria.

The constructs tpnP35S and tpnPTR1' were cloned as KpnI-PstI from their respective pUCSS plasmids into pUC19 containing the ampicillin resistance gene. The employed pMH10 derivatives were: pMH10Ds15GUS, pMH10Ds34CAT, pMH10Ds12IAA and pMH11Ds4IPTkan and are all carriers of a spectinomycin resistance gene. The cointegrate of the recombinant plasmids pMH11Ds4IPTkan and pUCSS19Ac9P35S is shown in Fig. 13 by way of example. These cointegrate plasmids were transmitted by transformation to the strain HB101 and after selection for ampicillin and spectinomycin they were characterized by plasmid preparation and restriction enzyme analysis. Then the plasmids were transmitted by conjugation to Agrobacterium, as described before.

The resulting Agrobacterium strains are an example of the use shown in Fig. 1.

The resulting recombinant Agrobacterium strains were tested on tobacco for expression of respectively nopaline synthase (pGV3850 marker), uidA, cat, root formation and shoot

formation and finally for transposition, before they were used on cereals.

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Claims

1. A process for the gene manipulation of cells of plants which, in essence, cannot be transformed by Agrobacterium tumefaciens with integration by T-DNA, which comprises introducing foreign DNA into the plant cells by infecting the plant cells with one or more recombinant Agrobacterium tumefaciens strains which contain foreign DNA to be transferred to the plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and are capable of transferring this foreign DNA to the plant cells, the foreign DNA located between the left- and right-hand ends of T-DNA comprising:
(A) a transposase gene located in an expression cassette active in the plant cells but not between the left- and right-hand transposon ends necessary for integration in the DNA of the plant cells, and
(B) a recombinant transposon comprising the left- and right-hand transposon ends necessary for integration in the DNA of the plant cells with an intermediate DNA fragment to be integrated in the DNA of the plant cells, but not comprising a DNA fragment which can lead in the plant cells to expression of an active transposase.

2. A process according to claim 1, which comprises the gene manipulation of plant cells of cereals such as maize, wheat, barley, rye, oat, rice etc., grasses such as ryegrass and fescue, leguminous plants such as leek, onion and garlic, ornamental plants such as tulipe, hyacinth etc. and other plant species such as conifer etc.

3. A process according to claim 1 or 2, which comprises using a genomic transposase gene.

4. A process according to claim 3, which comprises replacing the natural promoter of the genomic transposase gene by another promoter which can give expression in plant cells from which germinative cells develop.

5. A process according to any of the preceding claims, characterized in that between the left- and right-hand transposon ends the recombinant transposon comprises at least one foreign gene located in an expression cassette active in the plant cells.

6. A process according to any of the preceding claims, characterized in that also one or more selectable marker genes suitable for selection of transformed bacteria and located in an expression cassette active in bacteria are located between the left- and right-hand ends of T-DNA.

7. A process according to claim 6, characterized in that one or more selectable marker genes suitable for selection of transformed bacteria and located in an expression cassette active in bacteria form part of the recombinant transposon.

8. A process according to any of the preceding claims, characterized in that also one or more selectable marker genes suitable for selection of transformed plant cells and located in an expression cassette active in the plant cells are located between the left- and right-hand ends of T-DNA.

9. A process according to claim 8, characterized in that one or more selectable marker genes suitable for selection of transformed plant cells and located in an expression cassette active in the plant cells form part of the recombinant transposon.

10. A process according to any of the preceding claims, characterized in that the plant cells are infected with a mixture of two different recombinant A. tumefaciens strains, one of which comprises between the left- and right-hand ends of T-DNA the transposase gene located in an expression cassette active in the plant cells and the other comprises the recombinant transposon between the left- and right-hand ends of T-DNA.

11. A process according to claim 10, characterized in that between the T-DNA ends each of both strains contains at least one marker gene for selection of transformed bacteria

and at least one marker gene for selection of transformed plant cells.

12. A process according to claim 11, characterized in that between the T-DNA ends both strains each contain at least one different marker gene for selection of transformed plant cells.

13. A process according to any of claims 1-9, characterized in that the plant cells are infected with one recombinant A. tumefaciens strain which comprises between the left- and right-hand ends of T-DNA both the transposase gene located in an expression cassette active in the plant cells and the recombinant transposon.

14. A process according to claim 13, characterized in that the strain between the T-DNA ends contains at least one marker gene for selection of transformed bacteria and at least one marker gene for selection of transformed plant cells.

15. A process according to any of the preceding claims, characterized in that meristematic or embryogenic cells or tissues are infected with the bacteria.

16. A process according to claim 15, characterized in that cells of the apical meristem in germinating seeds are infected with the bacteria.

17. A process according to any of claims 15-16, characterized in that the infection is carried out by means of needleless high-pressure injection.

18. A process according to any of claims 15-16, characterized in that the infection is carried out by means of microinjection.

19. A recombinant plasmid consisting of a bacterial vector plasmid and at least one DNA insert comprising a transposase gene located in an expression cassette active in plant cells, which expression cassette active in plant cells and filled with a transposase gene is not located between the left- and right-hand transposon ends necessary for integration in the DNA of plant cells.

20. A recombinant plasmid according to claim 19, characterized in that it contains a genomic transposase gene.

21. A recombinant plasmid according to claim 20, characterized in that the natural promoter of the genomic transposase gene is replaced by another promoter which can give expression in plant cells from which germinative cells develop.

22. A recombinant plasmid consisting of a bacterial vector plasmid and at least one DNA insert comprising a recombinant transposon which contains the left- and right-hand transposon ends necessary for integration in the DNA of plant cells but no DNA fragment which can lead in plant cells to expression of an active transposase.

23. A recombinant plasmid according to claim 22, characterized in that between the left- and right-hand transposon ends the recombinant transposon comprises one or more restriction endonuclease recognition sequences suitable as cloning site.

24. A recombinant plasmid according to claim 22 or 23, characterized in that between the left- and right-hand transposon ends the recombinant transposon comprises at least one foreign gene located in an expression cassette active in plant cells.

25. A recombinant plasmid according any of claims 22-24, characterized in that the recombinant transposon comprises between the left- and right-hand transposon ends one or more selectable marker genes suitable for selection of transformed plant cells and located in an expression cassette active in plant cells.

26. A recombinant plasmid according any of claims 22-25, characterized in that that the recombinant transposon comprises between the left- and right-hand transposon ends one or more selectable marker genes suitable for selection of transformed bacteria and located in an expression cassette active in bacteria.

27. A recombinant Agrobacterium tumefaciens strain, which contains foreign DNA to be transferred to the plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and is capable of transferring this foreign DNA to the plant cells, characterized in that between the left- and right-hand ends of T-DNA a transposase gene is located in an expression cassette active in plant cells, which expression cassette active in plant cells and filled with a transposase gene is not located between the left- and right-hand transposon ends necessary for integration in the DNA of plant cells.

28. A recombinant Agrobacterium tumefaciens strain according to claim 27, characterized in that the transposase gene is a genomic transposase gene.

29. A recombinant Agrobacterium tumefaciens strain according to claim 28, characterized in that the natural promoter of the genomic transposase gene is replaced by another promoter which can give expression in plant cells from which germinative cells develop.

30. A recombinant Agrobacterium tumefaciens strain, which contains foreign DNA to be transferred to plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer to the plant cells and is capable of transferring this foreign DNA to the plant cells, characterized in that between the left- and right-hand ends of T-DNA a recombinant transposon is located which comprises the left- and right-hand transposon ends necessary for integration in the DNA of plant cells but contains no DNA fragment which can lead in plant cells to expression of an active transposase.

31. A recombinant Agrobacterium tumefaciens strain according to claim 30, characterized in that between the left- and right-hand transposon ends the recombinant transposon comprises at least one foreign gene located in an expression cassette active in plant cells.

32. A recombinant Agrobacterium tumefaciens strain according to claim 30 or 31, characterized in that the

recombinant transposon comprises between the left- and right-hand transposon ends one or more selectable marker genes suitable for selection of transformed plant cells and located in an expression cassette active in plant cells.

33. A recombinant Agrobacterium tumefaciens strain according any of claims 30-32, characterized in that the recombinant transposon comprises between the left- and right-hand transposon ends one or more selectable marker genes suitable for selection of transformed bacteria and located in an expression cassette active in bacteria.

34. A recombinant Agrobacterium tumefaciens strain according any of claims 30-33, characterized in that between the left- and right-hand ends of T-DNA there is also present a transposase gene located in an expression cassette active in plant cells, which expression cassette active in plant cells and filled with a transposase gene is not located between the left- and right-hand transposon ends necessary for integration in the DNA of plant cells.

35. Plants and parts consisting of one or more cells or products thereof, which plants derive from plant cells transformed by the process according to any of claims 1-18.

36. A process for the gene manipulation of cells of plants which, in essence, cannot be transformed by Agrobacterium tumefaciens with integration by T-DNA, which comprises introducing foreign DNA into the plant cells by infecting the plant cells with a recombinant Agrobacterium tumefaciens strain which contains foreign DNA to be transferred to the plant cells between the left- and right-hand ends of T-DNA necessary for such a transfer and is capable of transferring this foreign DNA to the plant cells, characterized in that plant cells are infected which contain one or more inactive transposons but no active transposase gene and that the foreign DNA located between the left- and right-hand ends of T-DNA comprises a transposase gene located in an expression cassette active in the plant cells, which expression cassette is not located between the left- and

right-hand transposon ends necessary for integration in the DNA of the plant cells.

37. A process according to claim 36, which comprises the gene manipulation of plant cells of cereals such as maize, wheat, barley, rye, oat, rice etc., grasses such as ryegrass and fescue, leguminous plants such as leek, onion and garlic, ornamental plants such as tulipe, hyacinth etc. and other plant species such as conifer etc.

38. A process according to claim 36 or 37, which comprises using a genomic transposase gene.

39. A process according to claim 38, which comprises replacing the natural promoter of the genomic transposase gene by another promoter which can give expression in plant cells from which germinative cells develop.

FIG.1

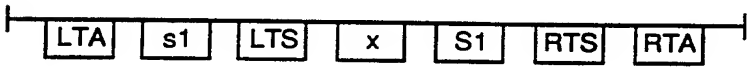

FIG.2a

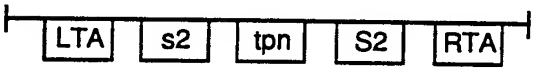

FIG.2b

FIG.3a

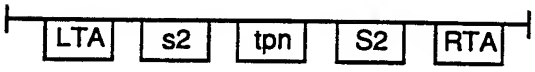

FIG.3b

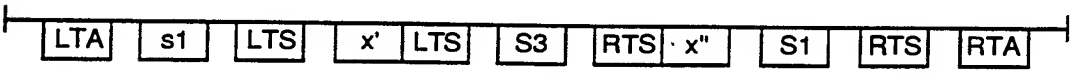

FIG.4a

FIG.4b

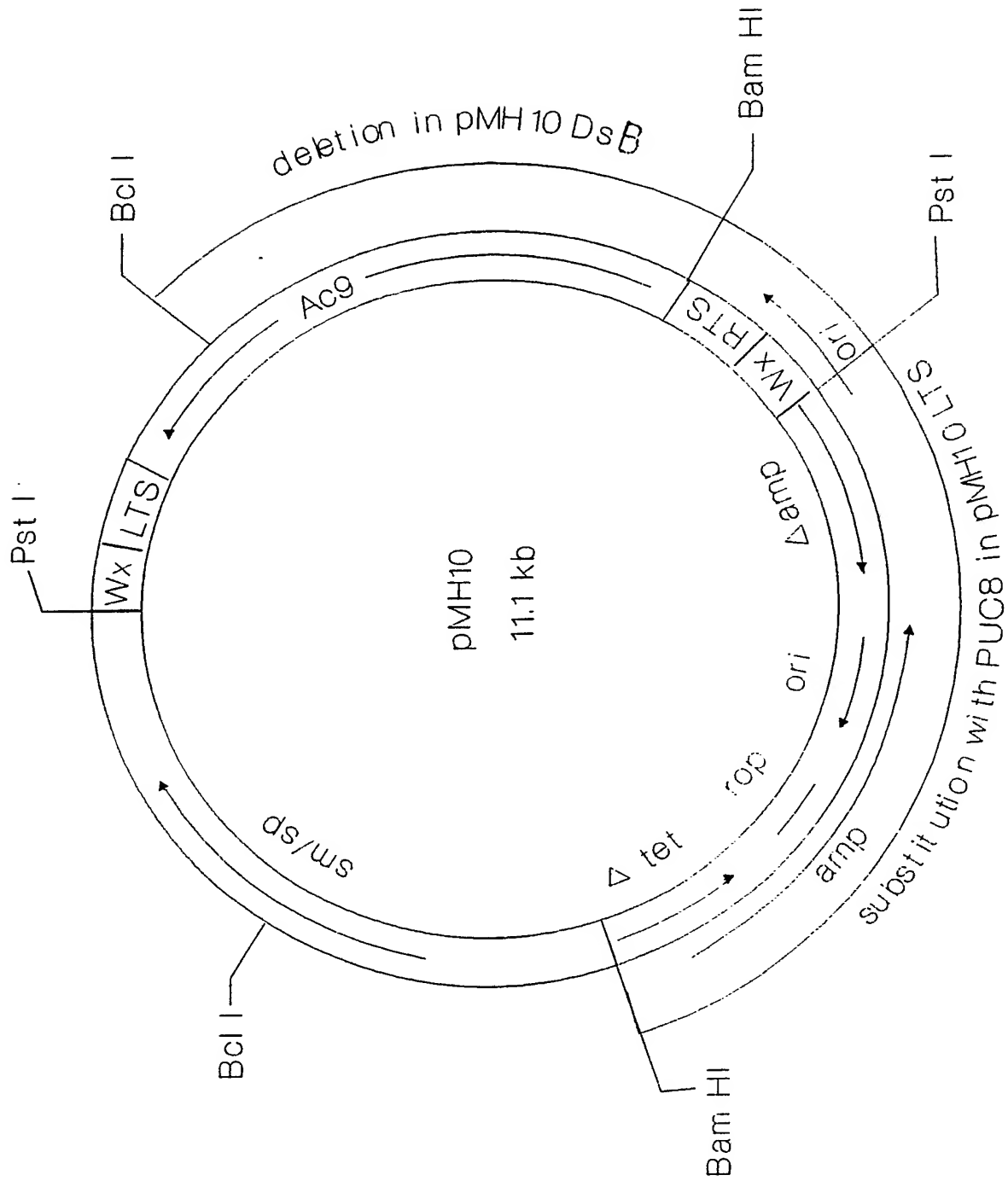
deletion in pMH10 DsB
Bam HI
Pst I
Bcl I
Ac9
RTS
Wx
ori
LTS
Wx
Pst I
pMH10
11.1 kb
Δamp
ori
rop
Δ tet
amp
ds/ws
Bcl I
Bam HI
substitution with PUC8 in pMH10 LTS

FIG.5a

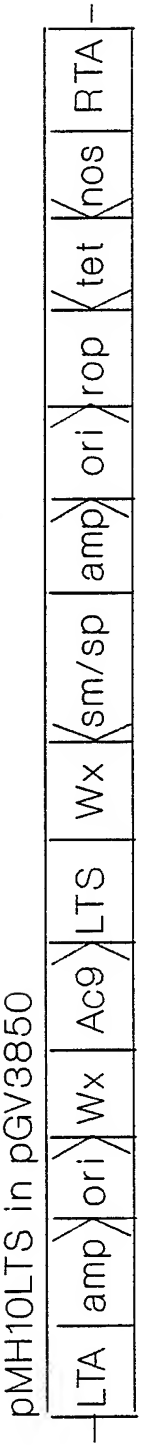
pMH10LTS in pGV3850
LTA
amp
ori
Wx
Ac9
LTS
Wx
sm/sp
amp
ori
rop
tet
nos
RTA

FIG.5b

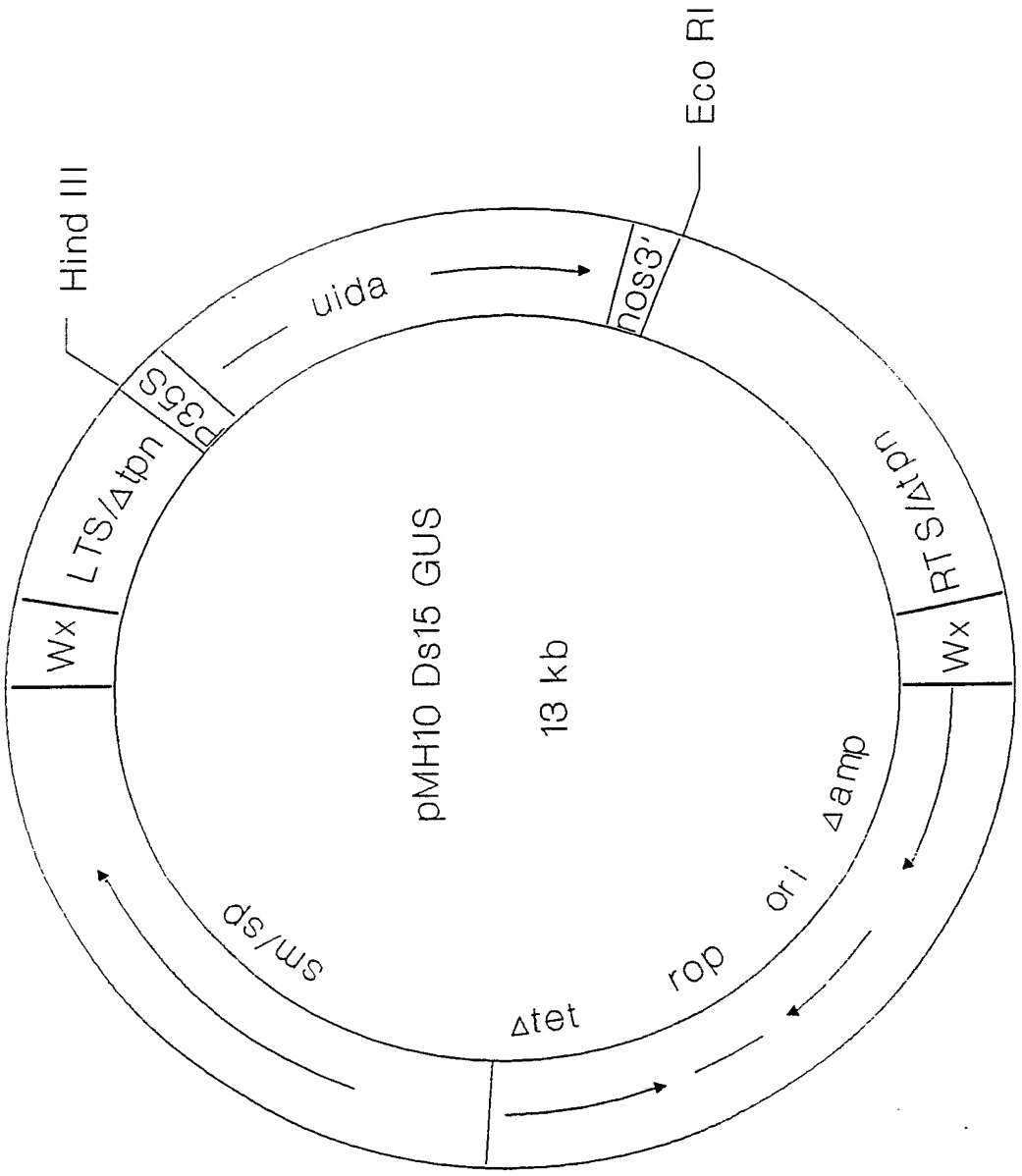
Eco RI
Hind III
uida
nos3'
35S
LTS/Δtpn
Wx
RTS/Δtpn
Wx
pMH10 Ds15 GUS
13 kb
Δamp
ori
rop
Δtet
sm/sp

FIG.6a

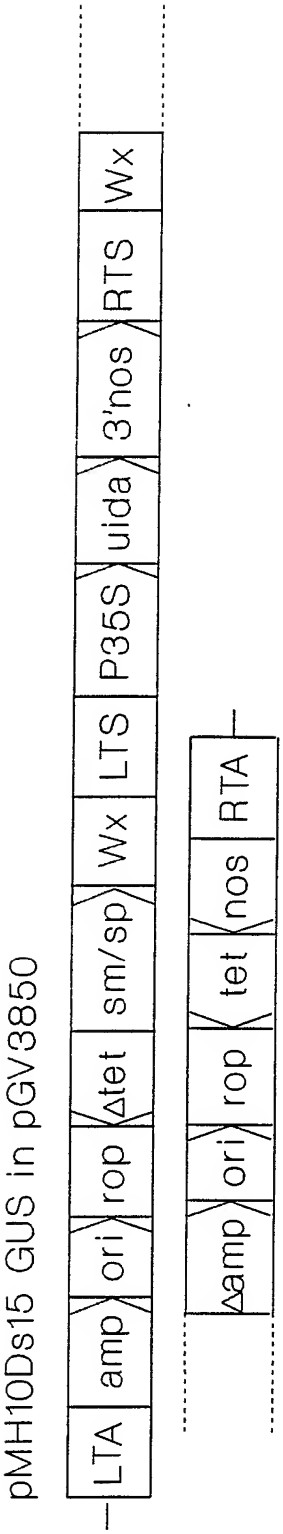
pMH10Ds15 GUS in pGV3850
LTA
amp
ori
rop
Δtet
sm/sp
Wx
LTS
P35S
uida
3'nos
RTS
Wx
Δamp
ori
rop
tet
nos
RTA

FIG.6b

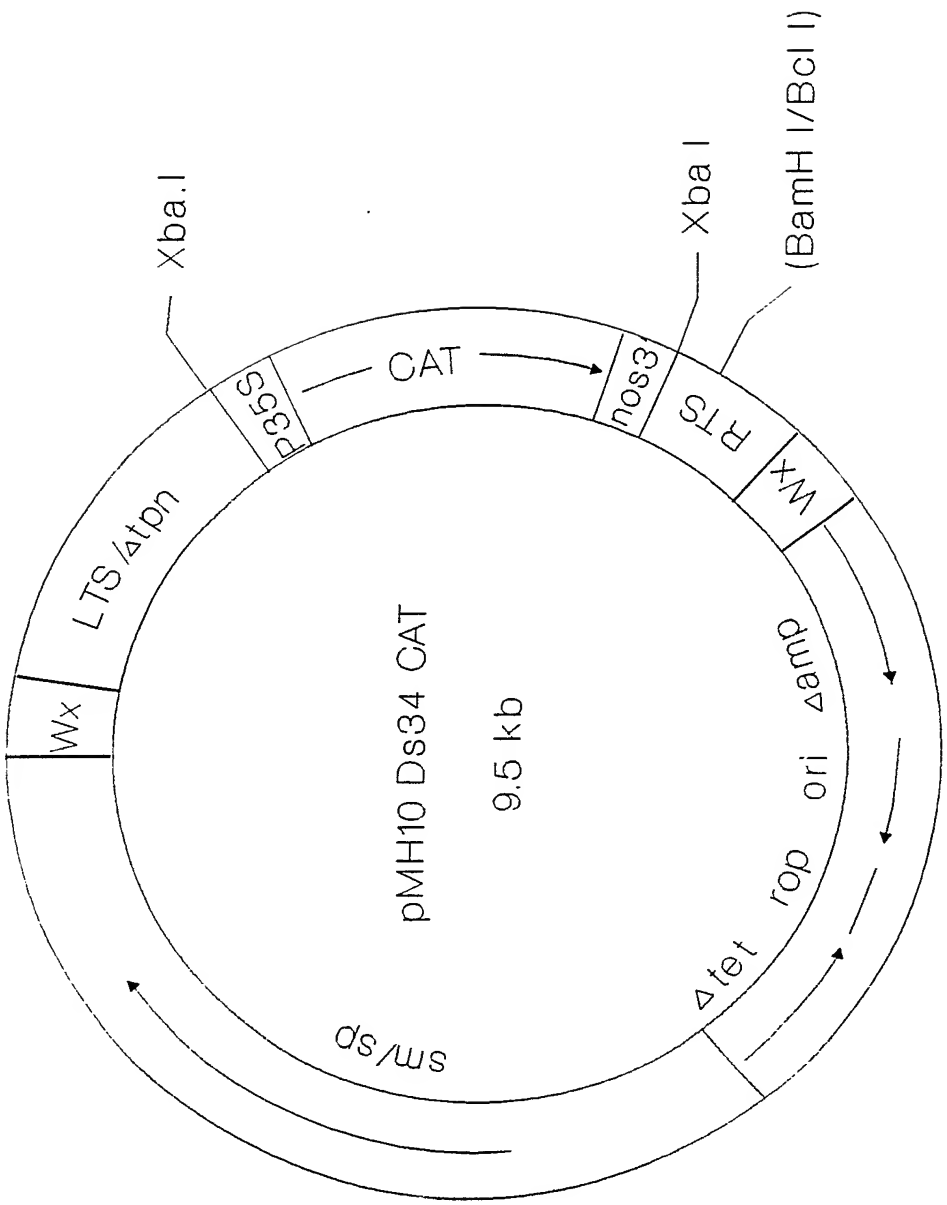
Xba.I
Xba I
(BamH I/Bcl I)
CAT
p35S
nos3'
RTS
Wx
LTS/Δtpn
Wx
pMH10 Ds34 CAT
9.5 kb
sm/sp
Δtet
rop
ori
Δamp

FIG.7

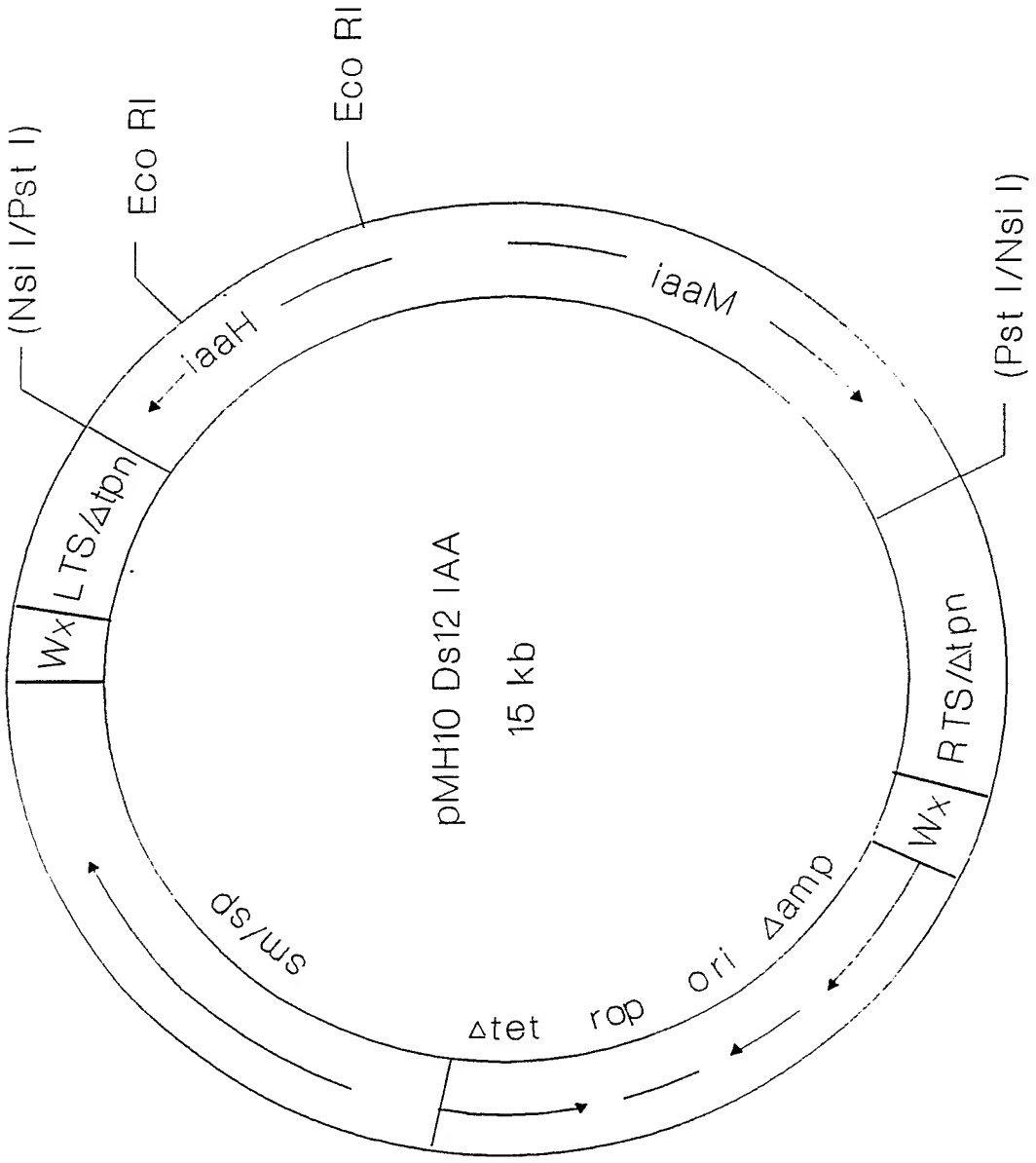
Eco RI
Eco RI
(Nsi I/Pst I)
(Pst I/Nsi I)
iaaH
iaaM
LTS/Δtpn
Wx
RTS/Δtpn
Wx
pMH10 Ds12 IAA
15 kb
ds/ws
sm/sp
Δtet
rop
ori
Δamp

FIG.8

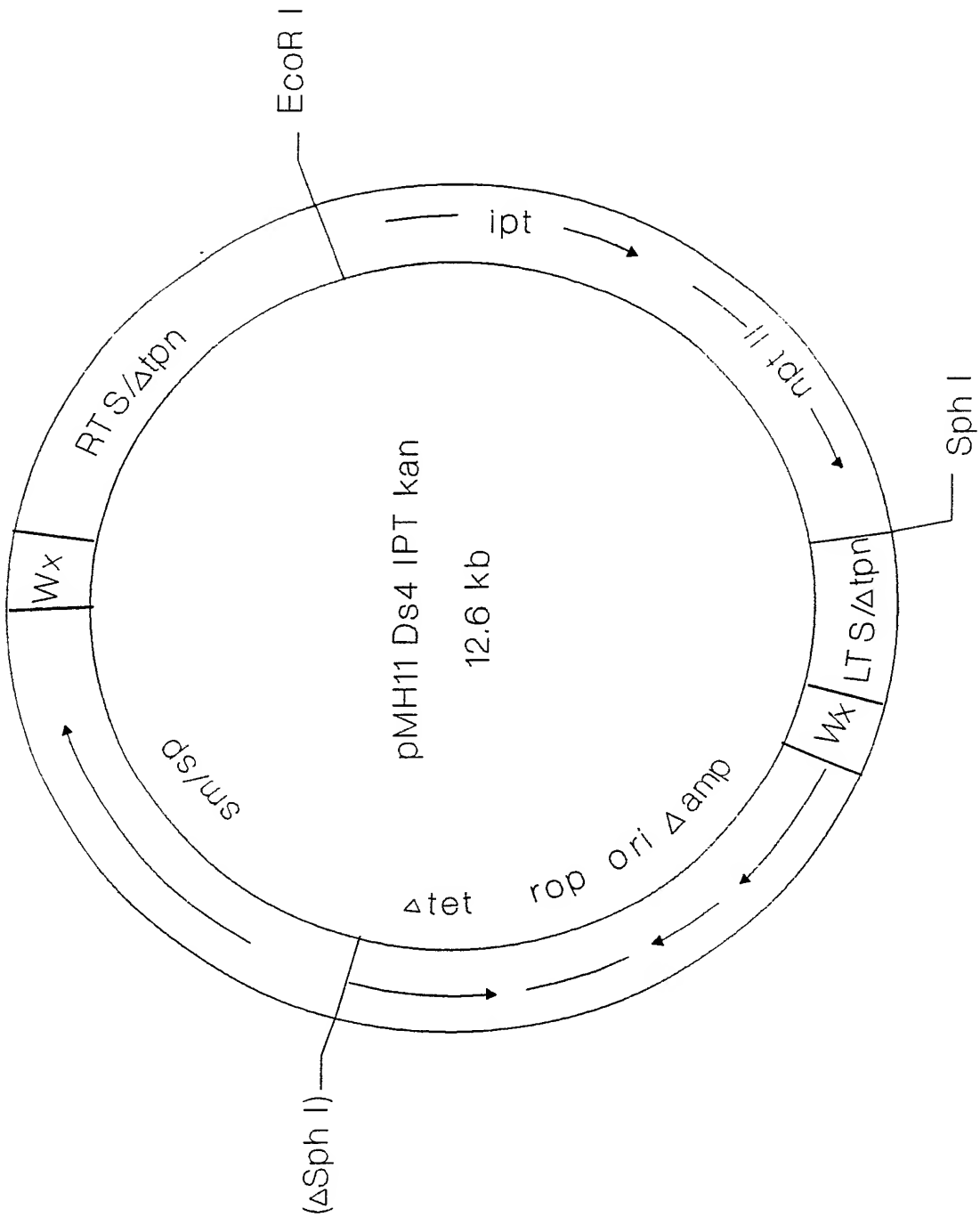
EcoR I
ipt
nptII
Sph I
LTS/Δtpn
Wx
rop ori Δamp
Δtet
sm/sp
(ΔSph I)
Wx
RTS/Δtpn
pMH11 Ds4 IPT kan
12.6 kb

FIG.9

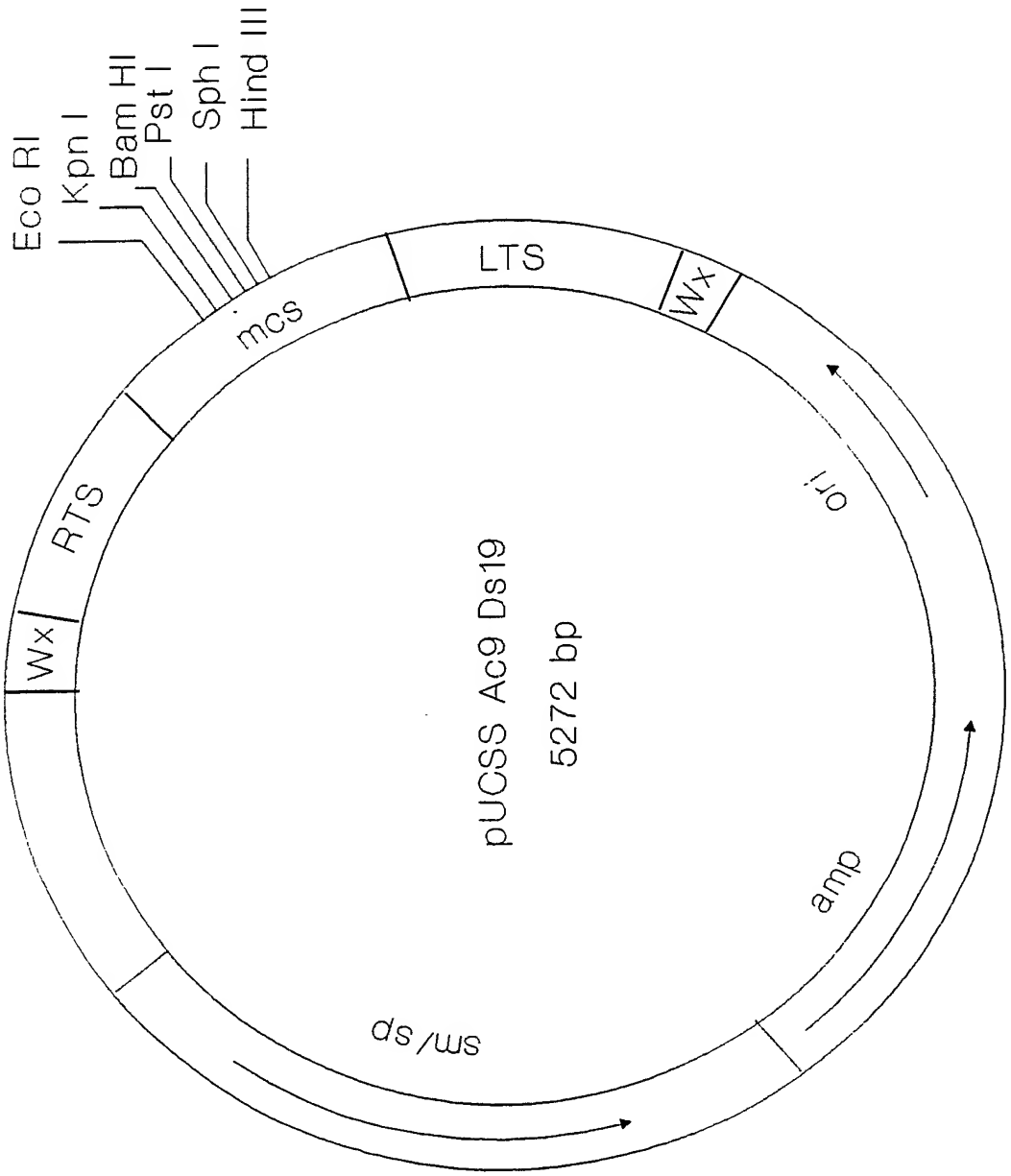
Eco RI
Kpn I
Bam HI
Pst I
Sph I
Hind III
mcs
LTS
Wx
ori
amp
sm/sp
RTS
Wx
pUCSS Ac9 Ds19
5272 bp

FIG.10

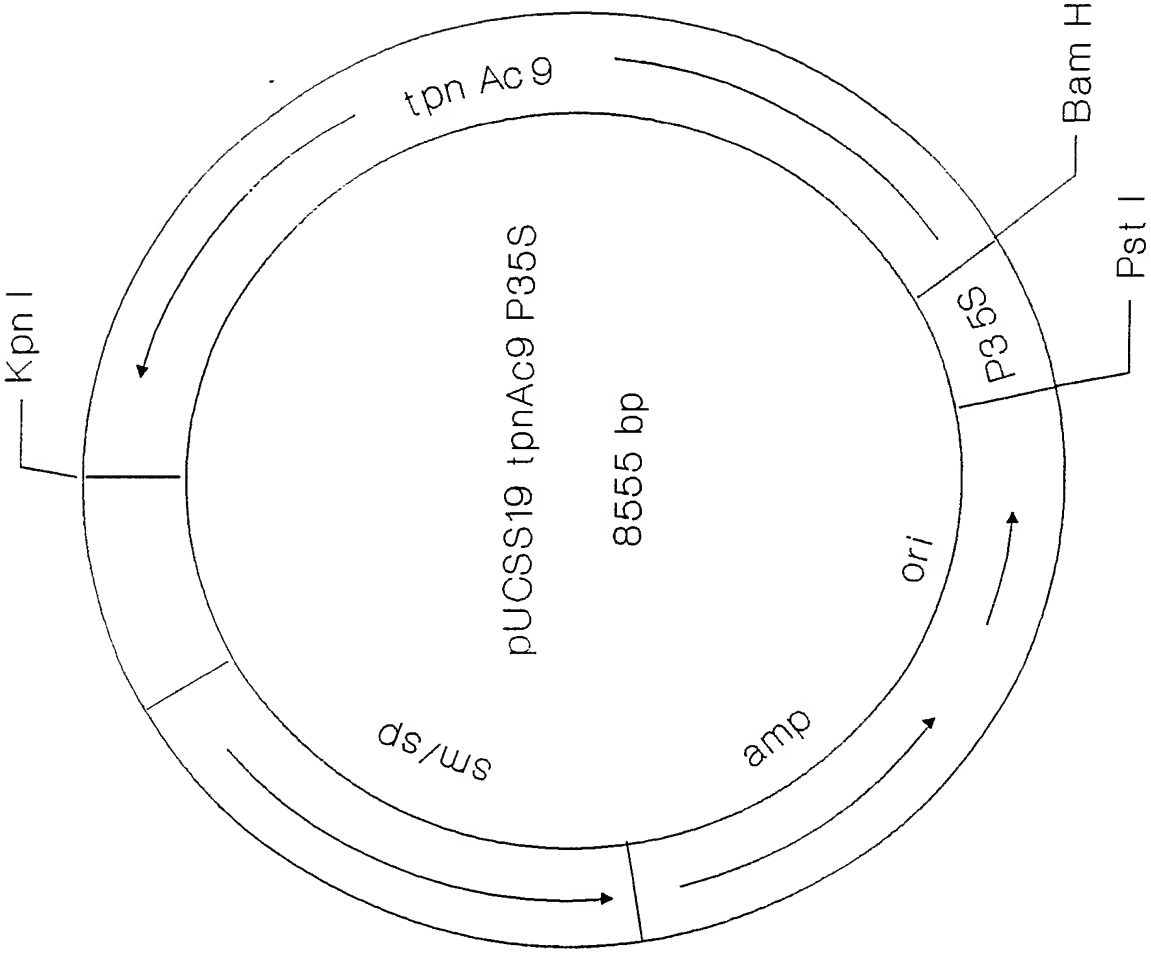
tpn Ac 9
Bam HI
Pst I
P35S
Kpn I
pUCSS19 tpnAc9 P35S
8555 bp
ori
ds/ws
amp

FIG.11a

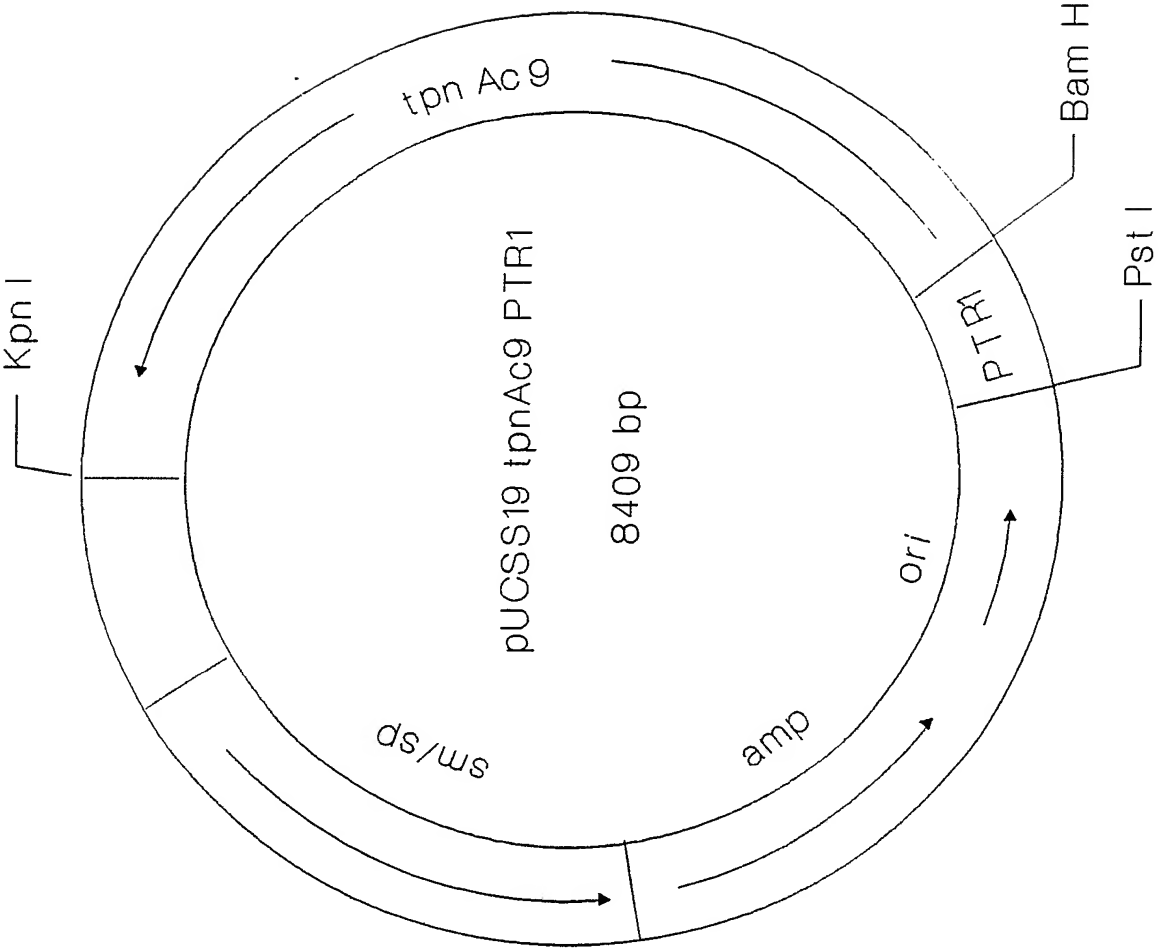
tpn Ac 9
Bam HI
Pst I
PTR1
Kpn I
pUCSS19 tpnAc9 PTR1
8409 bp
ori
ds/ws
amp

FIG.11b

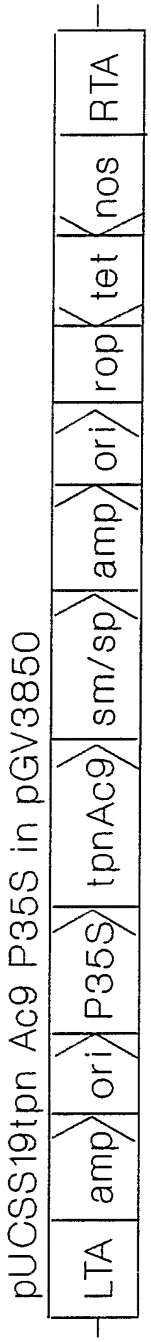
pUCSS19tpn Ac9 P35S in pGV3850
LTA
amp
ori
P35S
tpnAc9
sm/sp
amp
ori
rop
tet
nos
RTA

FIG.11c

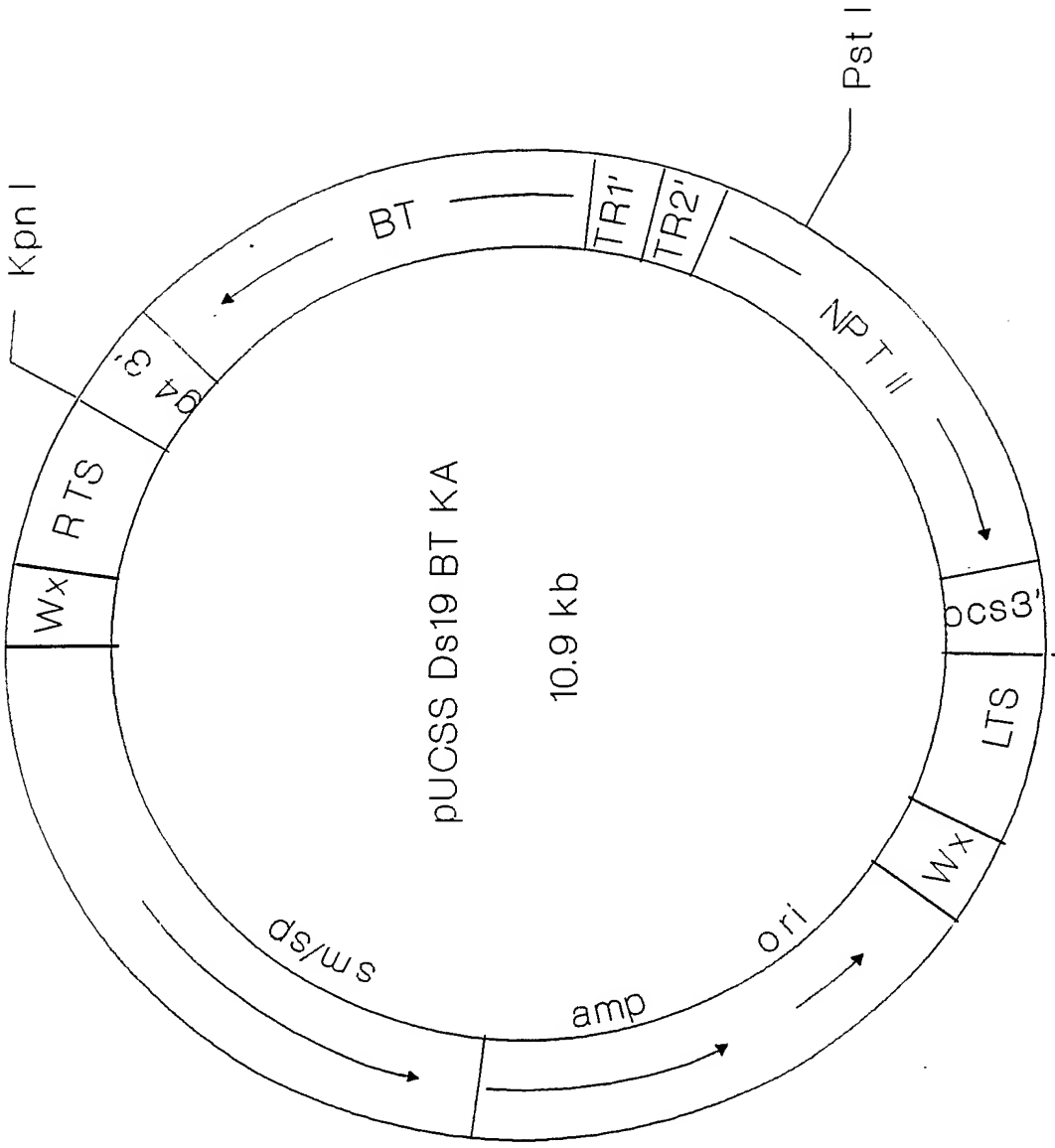
Pst I
Kpn I
BT
TR1'
TR2'
NPT II
9A 3'
RTS
Wx
pUCSS Ds19 BT KA
10.9 kb
ocs3'
Pst I
LTS
Wx
ds/ωs
amp
ori

FIG.12a

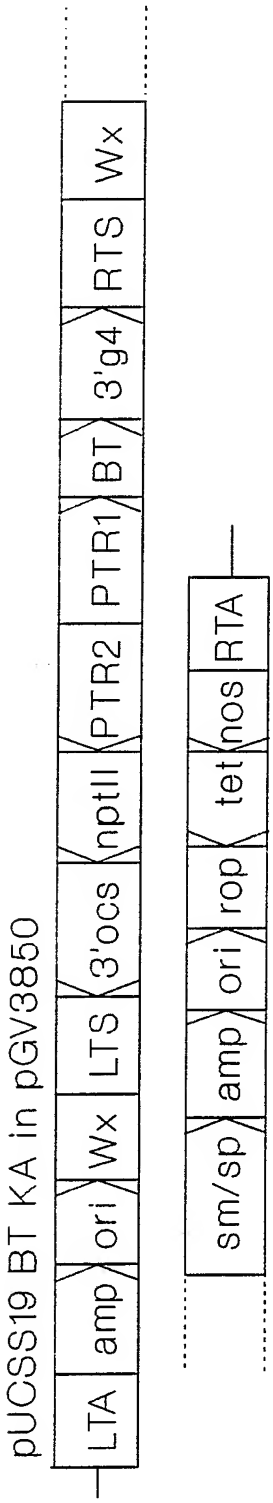
pUCSS19 BT KA in pGV3850
LTA
amp
ori
Wx
LTS
3'ocs
nptII
PTR2
PTR1
BT
3'g4
RTS
Wx
sm/sp
amp
ori
rop
tet
nos
RTA

FIG.12b

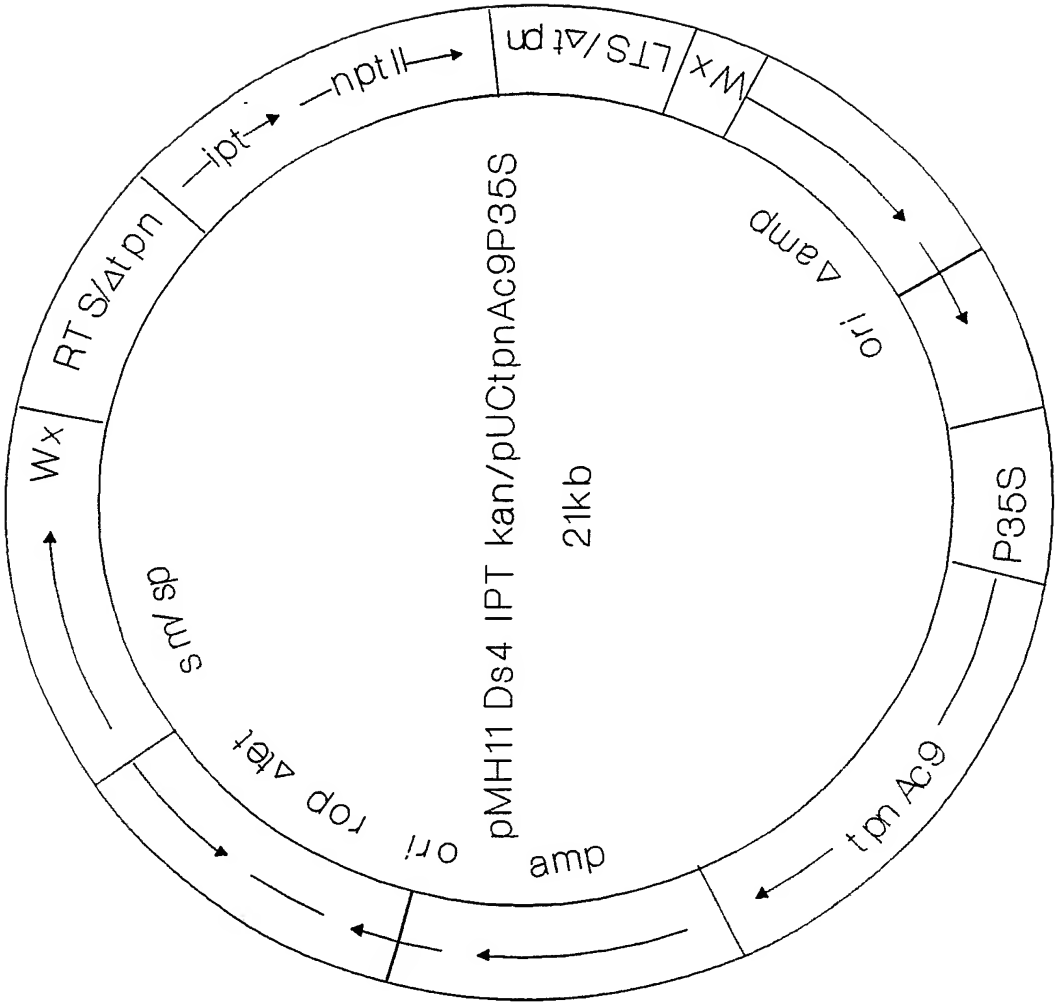
pMH11 Ds4 IPT kan/pUCtpnAc9P35S
21kb
Wx
LTS/Δtpn
Wx
ori Δamp
P35S
tpnAc9
amp
ori rop Δtet
sms
Wx
RTS/Δtpn
ipt
nptII

FIG.13

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/01883

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N1/21

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	MOL. GEN. GENET. vol. 218, 1989, pages 25 - 32; LASSNER, M. W.,ET.AL.: 'Genetic transactivation of Dissociation elements in transgenic tomato plants' see page 26-27 materials and methods: construction of the elements ---	19,20, 22,23, 26-28, 30,33
X	MOL. GEN. GENET. vol. 219, 1989, pages 461 - 466; MASTERSON, R. V., ET. AL.: 'A maize Ds transposable element containing a dihydrolate reductase gene transposes in Nicotiana tabacum and Arabidopsis thaliana' see materials and methods p461-462 and fig. 1 ,fig.2 --- -/--	19,20, 22-25, 27,28, 30-32

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 DECEMBER 1991	13. 12. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EMBO J. vol. 7, no. 12, 1988, pages 3653 - 3659; COUPLAND, G.,ET.AL.: 'Characterization of the maize transposable element Ac by internal deletions' see p. 3655 right col. para.1,fig.2, ---	19,2127, 29
X	NL,A,8 801 444 (SOLVAY) 2 January 1990 see page 5, line 34 - page 6, line 4; figure 3; example 1 ---	22-24, 30,31
A	EP,A,0 267 159 (CIBA-GEIGY) 11 May 1988 see claims and table 5 ---	15,16
A	WO,A,8 905 859 (UPJOHN) 29 June 1989 see page 8 ---	18
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, April 1989, WASHINGTON US pages 2219 - 2223; MASSON, P.,ET AL.: 'Mobility of the maize suppressor-mutator element in transgenic tobacco cells' see abstract, p.2222 left col, table2. ---	13,34
A	BIOLOGICAL ABSTRACTS BR40:14543 & CELL CULTURE AND CELL SOMATIC GENETICS OF PLANTS: MOLECULAR BIOLOGY OF PLANT NUCLEAR GENES vol. 6, 1990, EDS SCHELL, J. AND I. K. VASIL pages 101 - 132; FEDOROFF, N. V., ET AL.: 'Plant gene vectors and genetic transformation the structure function and uses of maize transposable elements' see the abstract ---	1-39
A	US,A,4 732 856 (FEDOROFF) 22 March 1988 see column 24, line 18 - line 34 ---	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9101883 SA 51689

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 04/12/91

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
NL-A-8801444	02-01-90	None	
EP-A-0267159	11-05-88	AU-B- 611652	20-06-91
		AU-A- 8089387	12-05-88
		JP-A- 63141590	14-06-88
WO-A-8905859	29-06-89	AU-A- 2818789	19-07-89
		EP-A- 0397687	22-11-90
US-A-4732856	22-03-88	None	